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~~PURIFICATION OF PSEUDANABAENA CATENATA EMDT.~~

ISOLATION OF FUNCTIONALLY FILAMENTS OF PSEUDANABAENA CATENATA AND OTHER PLANKTONIC CYANOBACTERIA

University — Université

UNIVERSITY OF ALBERTA.

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

MSc PLANT PHYSIOLOGY.

Year this degree conferred — Année d'obtention de ce grade: 1983

Name of Supervisor — Nom du directeur de thèse: P. R. Gorham.

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THE UNIVERSITY OF ALBERTA

The isolation of functionally axenic filaments of *Pseudanabaena catenata* and other planktonic cyanobacteria

by

Sean Mc Nicholas

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Plant Physiology

Botany

EDMONTON, ALBERTA

Fall 1983

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Sean Mc Nicholas

TITLE OF THESIS The isolation of functionally axenic filaments of *Pseudanabaena catenata* and other planktonic cyanobacteria

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

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Date October 3, 1983

Abstract

Whilst attempting, unsuccessfully, to isolate a type-C toxic strain(s) of *Aphanizomenon flos-aquae* from blooms collected from Lac La Nonne, Alberta, 67 out of the 73 colony isolates that grew were "taken-over" by different size and colour forms of another cyanobacterium, *Pseudanabaena catenata* Lauterb. Two of these *Pseudanabaena* cultures showed convulsant activity when injected intraperitoneally into mice.

Attempts to isolate the convulsant-producing strain failed. Five of the major Gram-negative bacterial contaminants were also tested and showed no convulsant activity.

Four widely cited methods for obtaining axenic strains were unsatisfactory when tested with *P. catenata*. Attempts were made to devise a more reliable procedure based on gentle washing of filaments rather than selective elimination of contaminants by treatment with chemicals or radiation. As few as three gravity washes with culture medium using Millipore or Nucleopore membranes caused extensive stasis or lysis. These effects were overcome by the addition of such colloids as dextran, polyvinylpyrrolidone and Ficoll to the washing medium. Optimum protection was provided by 1% dextran (MW 81,600). *P. catenata* withstood 10 washes with dextran which reduced the bacterial count from 15×10^6 to 15 per ml. After washing with dextran, single filament isolates were made from

overlays on a washed agar containing minerals and tolerable concentrations of vitamins and peptones (called P-2 Agar). Isolates were tested for contamination by streaking and incubating, for one month or longer, on Plate Count Agar, Nutrient Agar, Trypticase Soya Broth Agar and a highly enriched agar called P-3 Agar +soil extract at different pH's and temperatures.

Eleven functionally axenic strains of *P. catenata* were obtained by this procedure. Each strain exhibited a greater pH tolerance after the contaminating bacteria had been greatly reduced or eliminated from the culture medium. With slight modifications, this dextran washing and testing method has been successfully used to purify strains of *Aphanizomenon flos-aquae and Anabaena sub-cylindrica*.

Acknowledgements

The successful completion of this thesis was made possible by the assistance of several people and the University of Alberta.

First and foremost I would like to thank my supervisor Dr. Paul Gorham for his excellent ideas and guidance. Dr. M. Pickard and Dr. M. Hickman deserve thanks for their valuable discussion and constructive criticisms. I would also like to thank Judy Delange for her assistance during innumerable plating procedures and her ability to raise a smile at any situation. The assistance of Mike Folsom and Dr. D.D. Cass with the scanning electron microscope and Nomarski microscope observations, respectively, is gratefully acknowledged.

Finally I would like to thank my parents whose support, both emotionally and financially, made my study in Canada possible.

A Graduate Teaching Assistantship awarded by the Department of Botany, University of Alberta, and an operating grant from the National Science and Engineering Council provided financial support for this MSc. program.

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1. Introduction

Cyanobacterial toxins have long been associated with the death of livestock and wildlife in several countries. These toxins include hepatic toxins (peptides), neurotoxic alkaloids and lipopolysaccharides. The most recent reviews on toxic freshwater cyanobacteria are by Collins (1978), Gorham and Carmichael (1979), Carmichael and Gorham (1979), and the proceedings of an international conference on 'The Water Environment: Algal Toxins and Health' (1981). Section 2.1 presents a survey of the literature on the subject.

Aphanizomenon flos-aquae L. Ralfs. has been implicated in many poisonings (Sawyer *et al.* 1968). Recently, a strain has been found that releases a powerful neuromuscular blocking agent whose properties are similar to those of saxitoxin (Schantz *et al.* 1975), the paralytic shellfish poison produced by the marine dinoflagellates *Gonyaulax catenella* Kofoid and *G. tamarensis* Lebour.

Gorham *et al.* (1982) (Appendix I) found that during the summers of 1979 and 1980, blooms dominated by *Aphanizomenon flos-aquae* from Lac La Nonne (80 km. NW of Edmonton, Alberta) exhibited *Microcystis* type-*c* toxicity to mice (Carmichael and Gorham 1978) and agglutinated red blood cells (Carmichael and Bent 1981). This suggested the possibility that there were type-*c* toxic strains of *Aphanizomenon flos-aquae* in the lake. A search for type-*c* toxic strains of *Aphanizomenon* from this lake was undertaken by making a total of 128 colony isolates from five blooms

collected at various times throughout the summers of 1980 and 1981. Only 73 of the 128 colony isolates survived one month later. Of these, six were *Aphanizomenon* and all were non-toxic. The remaining 67 had been completely taken over by a small-celled species of filamentous cyanobacterium that most closely resembled *Pseudanabaena catenata* Lauterb. Among the 67 "take-over"cultures, two were found that produced violent convulsions but no type-*C* activity when injected intraperitoneally into mice. One of these two convulsant-producing cultures had haemagglutination activity. This demonstrated that haemagglutination and type-*C* activity, as observed by Carmichael and Bent (1981), were not invariably linked.

To stabilize growth and toxicity of a new strain of cyanobacterium and establish the source of toxin it is necessary to achieve an axenic, or more realistically, a functionally axenic state. A functionally axenic strain is defined as one having no demonstrable multiplying comtaminants but possibly harbouring one or a few contaminating cells in a state of stasis under the prevailing cultural conditions.

Attempts were made to isolate a functionally axenic strain of toxic *P. catenata* using many of the published methods mentioned in the literature survey in section 2.2, but the results were unsuccessful. From one of the toxic cultures, 14 non-axenic single-filament isolates of *P. catenata* were obtained but all were non-toxic. At the same

time it was found that both of the culture lines that originally had convulsant activity were no longer toxic, presumably because of selection and/or take-over by non-toxic components during subculturing. Attempts were made to recover the convulsant activity by going back for inocula to old subcultures that had been retained. This, too, was unsuccesful. Further attempts to isolate a functionally axenic strain of toxic *P. catenata* were abandoned.

Four widely cited methods for obtaining functionally axenic cultures, ^{60}Co gamma radiation (Kraus 1969,1976), sulphide gradient (Parker 1982), antibiotics (Vaara and Vaara 1979, Rippka 1979) and phenol (Carmichael and Gorham 1974) were examined using a strain of *P. catenata* and other planktonic cyanobacteria. Since none of these methods gave satisfactory results, effort was concentrated on improving the washing and spatial separation techniques of Carmichael and Gorham (1974). Two gentler washing procedures were developed. However, washed filaments in nutrient medium exhibited stasis, clipping and lysis within a few hours after suspensions with low bacterial counts were mixed with equal volumes of washed agar medium and poured into Petri dishes. Unwashed filaments plated in the same way grew normally, albeit in the presence of high numbers of bacterial colonies. No improvement was achieved by washing with used medium that had first been rendered sterile by filtration through a 1.2-µm Millipore membrane.

As a follow-up to this published work (Gorham *et al*,1982) (Appendix I), it was decided to examine two particular aspects. One was the question of whether or not the bacteria were involved in the production of toxin by one of the cultures of *P. catenata* that exhibited convulsant activity. The approach was to isolate and mass culture the principal contaminants and test them for toxicity by intraperitoneal injection of extracts into mice. The other aspect was to critically examine the four previously mentioned methods for obtaining axenic cultures and attempt to adapt and improve the ones that appeared the most promising for use with *P. catenata* and other planktonic cyanobacteria.

High-molecular-weight colloids of low osmotic potential have been used for many years in the isolation of delicately-membraned cells and organelles in both plant and mammalian physiology (Honda *et al*. 1966, Allfrey *et al*. 1964). Workers have suggested that these colloids provide protection to the membranes, shielding them from the physical and osmotic stresses incurred during isolation. Selected grades of these colloids were, therefore,added to washing medium to determine if any would act as cell envelope protectants for the cyanobacterial filaments.

In preliminary work it was found that the inclusion of 1% Polyvinylpyrrolidone (PVP) (M.W.40,000) in the washing medium provided protection against cellular damage to a strain of *P. catenata* as judged by greatly increased

filament viability. A systematic study was, therefore, undertaken to examine this and other high-molecular-weight colloids for their value as protectants during gravity-washing of *P. catenata* and other species of planktonic cyanobacteria. The intention was to determine the optimum type, grade, molecular weight, concentration and mode of action of these compounds. The possibility that the failure of antibiotics and antiseptics could be reversed by the inclusion of high-molecular-weight colloids was also examined.

Attempts were then made to improve the media and methods for identifying the presence of contaminating bacteria and for retrieving from agar viable cyanobacterial colonies that were apparently bacteria-free. The improvements were then combined with the most successful washing procedure to produce a more reliable method for obtaining functionally axenic clones of *P. catenata* and other planktonic species than ever achieved before.

2. Literature Survey

2.1 Toxic cyanobacteria

Outbreaks of cyanobacterial poisonings have been documented world-wide since the late 1800's, with the majority of references appearing since the late 1940's. Of the several genera of cyanobacteria that have been implicated with the death of livestock, waterfowl and fish, toxic isolates have been cultured in only three. These are *Microcystis aeruginosa* Kutz. emend. Elenkin, (includes *Microcystis toxica* Stephens), *Anabaena flos-aquae* (Lyngb.) de Breb. and *Aphanizomenon flos-aquae* (L.) Ralfs. Sickness and possible death occurs when toxic strains of common species dominate the bloom sufficiently to provide above-threshold levels of phycotoxins to susceptible animals.

The early evidence of toxicity associated with cyanobacteria came exclusively from field observations. It was noted that livestock which watered on ponds with extensive cyanobacterial populations became ill and frequently died within a few hours of consumption. Francis (1878) reported that a thick scum of *Nodularia spumigena* on Lake Alexandria, Australia, was responsible for the deaths of sheep, dogs, horses and pigs. At about the same time Porter, Arthur *et al.* (1886) reported that *Gloeotrichia echinulata* was responsible for the deaths of domestic animals in Minnesota. Fitch *et al.*(1934) reported that a

bloom of *Coelosphaerium kuetzingianum* was responsible for mortalities of sheep, chickens and hogs in Oaks Lake, Minnesota during the summer of 1918, whilst the cattle deaths in Minnesota (1930-31) could be attributed to *Microcystis aeruginosa* and *Anabaena flos-aquae*. Steyn (1945) stated that *Microcystis toxica* was responsible for the death of thousands of sheep and cattle in the Vaal Dam region of South Africa since the turn of the century. South African workers headed by Louw (1950), however, were among the first to try to identify a phycotoxin released by a *Microcystis*-dominated bloom in the Vaal Dam. They concluded that the toxin was an hepatic alkaloid. This was later proved to be incorrect when Hughes *et al.*(1958) were able to obtain a toxic colony isolate, designated NRC-1, from a Canadian lake. The toxin, initially called Fast Death Factor(FDF), later renamed microcystin (Konst *et al.*1965), proved to be one of five, closely related, low-molecular-weight peptides having properties that suggested a cyclic structure (Bishop *et al.*1959). The toxin produced enlargement and congestion of the liver with necrosis of the hepatic cells and punctate haemorrhages that were constant and pathognomic (Konst *et al.*1965). More recently, Carmichael and Gorham (1978) have isolated strains of *Microcystis aeruginosa* from lakes in Alberta and Saskatchewan which produce signs of poisoning that differ from those of microcystin. Death is by cardiovascular collapse, with signs of some liver damage indicating a

two-fold nature to the toxin. Since the symptoms closely resemble those of *Anabaena* type-*c* toxin, it has been designated *Microcystis* type-*c* toxin.

Runnegar and Falconer (1982) investigated the effects of a toxin from a *Microcystis* bloom (that occurred in an Australian reservoir) on liver slices, isolated mitochondria, and microsomes from mice. The toxin affects hepatocytes *in vitro* in a specific way causing deformation in a dose-dependant manner. The peptide toxin was shown to have no significant influence on the major metabolic functions in the liver indicating it is a cellular rather than metabolic toxin.

Over the years, confusing results have been reported about the structure of microcystin and other toxic peptides produced by strains and blooms of *Microcystis aeruginosa*. Eloff *et al.* (1982), and Botes *et al.*(1982) have recently shown that one of the principal reasons for the variability is that different strains produce mixtures of chemically related toxic peptides (which can be resolved by high performance liquid chromatography). However, findings of the two groups differ in several important respects, particularly as to the molecular weight, amino acid composition and sequence, and whether or not there is a 20-carbon non-peptide moiety as part of each.

Anabaena flos-aquae has been studied as a toxic cyanobacterium by many workers including Firkins (1953), Rose (1953) and Olson (1960), but Gorham *et al.*(1964) were

the first to report a toxic colony isolate. The toxin, initially called Very Fast Death Factor (VFDF.), and later renamed anatoxin-*a* (Devlin *et al.*1977) is a powerful post-synaptic depolarizing neuromuscular blocking agent which causes death by respiratory arrest (Carmichael *et al.*1975). It is an alkaloid, 2-acetyl-9-azo-bicyclo (4.2.1.) non-2-ene, having a molecular weight of 165 (Devlin *et al.*1977). Two syntheses of anatoxin-*a* have been published (Campbell *et al.*1977, Bates and Rappaport 1979). It produces a post-synaptic depolarising sign of opisthotonous and muscular rigidity in avian species such as duck and chick. By this pharmacological sign and others, such as lachrymation, chromodachryorrhea (bloody tears) and salivation, and by different survival times with mice, rats and chicks it has been possible to identify three, and possibly five, additional anatoxins (Carmichael and Gorham 1978, Carmichael 1982) provisionally called anatoxins-*a(s)*,-*b*, -*b(s)*, -*c*, and -*d*.

The third major bloom-forming species, *Aphanizomenon flos-aquae*, has been associated with toxic outbreaks for many years, but until the late 1960's no toxic isolate had been recorded. Sawyer *et al.*(1968) isolated an atypical, non-colony-forming, non-axenic clone of the species from a New Hampshire lake. The toxin produced is called aphantoxin (Alam *et al.*1973). It has chemical and pharmacological properties which resemble some, if not all, of those possessed by saxitoxin (Schantz *et al.*1975, Schantz 1981)

which is produced by the marine dinoflagellates *Gonyaulax catenella*. and *G. tamarensis*. Work by Gentile and Maloney (1969) indicated that aphantoxin was released only from aged or lysed cells. More recently Alam *et al.*(1973,1978) and Alam and Euler (1981) demonstrated that aphantoxin consists of a mixture of saxitoxin and some of its derivatives, especially neosaxitoxin and gonyautoxin II (Carmichael, 1982).

Aphantoxin has recently been studied pharmacologically by Carmichael (1982). His work indicates that aphantoxin has agonist properties on chick biventer cervicus and guinea pig ileum muscle preparations. These results are in contrast to results with saxitoxin. The latter has been found to specifically block sodium channels (Gentile and Maloney 1969, Sasner Jr. *et al.*1981), and has little effect on these muscle preparations. Carmichael suggests that his results can be explained if aphantoxin has an effect on calcium as well as sodium channels.

There are reports of toxicity produced by several other species of cyanobacteria (Schwimmer and Schwimmer 1964, Carmichael 1982). Besides mammals and birds, invertebrates (Lambert 1981, Ransom *et al.*1978), true algae and other species of cyanobacteria (Mason *et al.*1982) may be affected.

The only reported incidence of toxicity concerning *Pseudanabaena* is that of a bloom of *Microcystis farlowiana* and *Pseudanabaena franquetti* from Lac du Bourget (Savoie) in France which was found to inhibit benthic fauna (Gevrey *et*

*al.*1972, Michel *et al.*1972). Experiments indicated that one or several toxic factors liberated by the death of the cyanobacterial cells killed fish, chironomid larvae and molluscs (species of Anodonta). The snails died within 48 hours, their death preceded by a general paralysis. The toxic factor(s) was thermoresistant and soluble in water with only the lysed cells exhibiting toxic action.

2.2 Isolation and Purification of Cyanobacteria

Past experience with studies of toxic cyanobacteria has demonstrated the need for obtaining axenic or, at least, functionally axenic strains to establish the source of the toxin and to help stabilize growth and toxicity of mass cultures. The primary obstacle to obtaining a functionally axenic culture lies in the successful elimination of the relatively high populations of free-living or associated bacteria without causing damage to the desired cyanobacterial cells (such as causing them to lyse or enter a period of prolonged stasis). Recent work by Ecker, Foxall and Sasner (1981) has provided a series of electron micrographs of glutaraldehyde-fixed filaments of three species of cyanobacteria that show bacteria adhering to the extracellular mucilagenous layer which is present with many species. Pearl and Kellar (1978), and Ecker *et al.*(1981), have indicated that bacteria associated with heterocysts of *Anabaena* and *Aphanizomenon* assimilate amino acids and sugars as well as increase the efficiency of nitrogen fixation. The

mature heterocyst wall in these species has been reported to contain more than 70% glucose compared with 35% in the vegetative cell (Dunn and Wolk,1970). These two factors combined with the nitrogen-fixing ability of heterocysts may account for the presence of bacteria attached to these cells. It is entirely conceivable, therefore, that some cyanobacteria are obligate symbionts (Ecker *et al*.1981).

Leppard *et al*.(1977) and Massalaski and Leppard (1979) have provided proof of the existence of electron-opaque, non-rigid microfibrils of approximately 3 to 10nm in diameter which are found in abundance in the outer cell envelope of certain species of freshwater algae, cyanobacteria and microbes. Individual fibres may form a complex mesh-like aggregate which can break apart and re-associate. Mesh-like aggregates also appear to adhere to cells, thereby further increasing the possibility of bacterial association.

The purification of cyanobacteria is essential to avoid possible complications associated with the presence of contaminating microorganisms and various methods have been employed by workers to reach this end.

Kraus (1969,1976) and Lange (1976) have reported that appropriate doses of gamma radiation will selectively eliminate a bacterial flora, leaving a population of viable, axenic cyanobacteria. Kraus reported a tri-level variation in species susceptibility to radiation, with the highest dosage, 1200krads, completely removing any bacterial

presence, whilst leaving the test species of cyanobacteria axenic and viable. However, it must be noted that only *Anacystis nidulans* and an *Oscillatoria sp.* were able to survive such high dosages. All planktonic forms were far more susceptible to radiation damage and lysis at dosages in excess of 300krads.

The use of antibiotics to obtain bacteria-free cultures of algae and cyanobacteria has been reported by several authors (Carmichael and Gorham 1974). Provasoli *et al.*(1951) found that the use of appropriate combinations of the antibiotics penicillin, streptomycin, chlorotetracycline, chloramphenicol, bacitracin and polymyxin resulted in the elimination of both Gram-positive and Gram-negative bacterial contaminants from algal and protozoan cultures. However, Tchan and Gould (1961) and Hunter and McVeigh (1961) reported that the inclusion of antibiotics in the culture medium of cyanobacteria proved to be more inhibitory to the cyanobacteria than the bacteria.

Vaara and Vaara (1979) have recently reported that the addition of the antibiotic, D-cycloserine, to a culture is an effective way of reducing its bacterial population. Results indicated that 13 strains of *Pseudanabaena* could effectively be termed axenic after treatment with cycloserine in tryptone-yeast extract-glucose broth (TYG) in the dark to select for obligate photoautotrophs. Purification of these strains employed the general principles of the classic antibiotic enrichment method,

originally developed for isolation of auxotrophic bacterial mutants (Hopwood, 1970). More recently, Rippka (1979) has also indicated that the antibiotic, ampicillin, can effectively be used to purify cyanobacterial strains following the procedure of Hopwood.

Parker (1982) has recently reported that *Microcystis* can be successfully treated with sodium sulphide and sulphite to selectively eliminate contaminating bacteria. This method employs a nutrient agar plate containing 2mM sodium sulphite upon which a drop of 2M sodium sulphide is placed at the centre and allowed to diffuse outwards. The complex radial gradients of pH, sulphide and sulphite provide a zone of bacterial suppression and lysis whilst allowing the cyanobacterial cells to retain viability.

The culturing of certain cyanobacteria on agar media has been employed by phycologists for many years. However, it has also been noted that certain planktonic forms could not be reliably grown on agar. Early work on agar culturing was carried out by M.B. Allen (1952) and M.M. Allen (1968). The latter discovered that the toxicity of agar to *Anacystis nidulans* could be reduced by autoclaving the agar and mineral nutrients separately. Stanier *et al.*(1971), utilized this method to successfully produce axenic cultures of non-motile cyanobacteria by early transfer of colonies performed under a dissecting microscope together with numerous restreakings to selectively eliminate the contaminating bacteria.

Carmichael and Gorham (1974) have reported that agar contains water-soluble inhibitors that can be removed by washing the agar in distilled water. They found that cyanobacterial colonies, developed from single filaments well isolated from bacterial colonies when poured in washed agar, could be selected by using an inverted microscope and removed in agar cores to sterile medium by use of a Pasteur pipette.

Allen and Gorham (1981) discovered that the toxicity in agar was generated by heat-induced chemical reactions. They found the titer of lytic agent(s) to be a function of temperature, duration and repetition of heating. The lytic agent(s) could be removed by repeated leachings with purified distilled water. Care must be taken to sterilize washed agar by germicidal UV radiation and to barely melt it at 70°C in a microwave oven to avoid the generation of a fresh batch of lytic agent(s).

Koch (1965) and Vaara and Vaara (1979) reported that some filamentous cyanobacteria have been purified by taking advantage of their vigorous gliding movements and inducing phototactic migration of trichomes. A small patch of material was placed on scored agar at one side of a Petri dish which was illuminated from the opposite side. The cyanobacteria responded by gliding across the plate towards the region of higher light intensity. As soon as some of the organisms reached the opposite side they were removed from the agar and subjected to a repetition of the same

procedure. The gliding movement of the cyanobacteria allowed some filaments to outgrow the bacterial contaminants resulting in an axenic culture. Vaara and Vaara (1979) claimed that by using this way they were able to purify species of the following genera: *Pseudanabaena*, *Oscillatoria* and *Anabaena*.

It has been found that phenol is more toxic to actively dividing bacterial cells than to non-dividing cells (Hobby *et al.*1942). Carmichael and Gorham (1974) based their method for obtaining axenic cultures on this differentiating effect of phenol. By treating suspensions of bacteria and cyanobacteria in a nutrient-rich medium in the dark, actively dividing heterotrophic bacterial cells become more susceptible to phenolic attack than the non-dividing cyanobacteria. After washing, dispersing in pour-plates prepared with washed agar and incubation, filaments were isolated by coring and tested for axenicity by phase-contrast microscopy and a battery of bacteriological media incubated at different temperatures.

Carmichael and Gorham (1974) and Gorham *et al.*(1982) have reported upon the use of washing processes to remove antibacterial agents or to serially dilute bacterial contaminants from a culture of cyanobacteria. Although serial dilution succeeded in greatly reducing the bacterial count it also caused some form of damage to the cell envelopes of the cyanobacteria, resulting in stasis and, more frequently, lysis.

In a variety of biological situations, inert non-ionized polymers of high molecular weight are utilized as protective colloids. Some rather well-defined substances with such properties are readily available. Most of these, however, even in moderate concentrations, yield highly viscous solutions which may be disadvantageous for some applications. Ficoll and Polyvinylpyrrolidone (PVP) have been used as stabilizing agents in protein solutions and in the preparation and purification of living cells and cell fragments (Honda *et al.*1966, Ketchum and Holt 1970). High molecular-weight polymers of D-glucopyranose, synthesized from sucrose by a number of bacterial species belonging to the family Lactobacillaceae and named dextrans, have also been used as protective colloids (Ponder 1957, Rampling and Sirs 1975, and Ogiso *et al.*1977). At high molecular weights these colloids do not permeate cell membranes and exhibit very little osmotic action. They have been used in the preparation of axenic, morphologically intact nuclei from spinach mesophyll cells (Honda *et al.*1966, Albertson 1971). Suggestions by previous workers, Reardon *et al.*(1979), have indicated that microalgae can successfully be isolated by centrifugation utilizing high molecular-weight colloidal substances. A greater incidence of membrane integrity was achieved after centrifugation, indicating that the colloid had acted as a "membrane-protectant" shielding the cells from the physical damage incurred during the separation process. In a follow-up to the work reported by Gorham *et*

al. (1982) stasis and lysis of cyanobacteria caused by gentle washing could be prevented by the inclusion of an appropriate concentration and molecular weight of PVP in the nutrient medium for washing.

3. Materials and Methods

3.1 Cyanobacterial cultures and their sources

The following cultures, isolated from Lac La Nonne, were used:

Pseudanabaena catenata Lauterb. A81-15(B1) and strains (single-filament isolates or re-isolates designated by appended letters, numbers, letters, such as -g-22-b)

Aphanizomenon flos-aquae Ralfs. A81-31(C5)

Other cultures used for purposes of comparison were:

Anabaena flos-aquae (Lyngb.) de Breb., NRC-44-1 (strain)

Anabaena sub-cylindrica Borge A78-1(strain)

Pseudanabaena catenata Lauterb. CCAP 1464/1

Pseudanabaena brunea (auct?) CCAP 1464/2

The last two cultures were obtained from the Culture Collection of Algae and Protozoa, Cambridge, England and were isolated by E.G. Pringsheim.

3.2 Identification of Pseudanabaena Isolates

The 67 *Aphanizomenon flos-aquae* cultures that were completely "taken-over" (Table 1) by a species of cyanobacterium that most closely resembled *Pseudanabaena catenata* Lauterb. (Geitler 1932) were examined macroscopically and microscopically with phase contrast and Nomarski optics.

Table 1. Colony isolates of Aphanizomenon blooms collected from Lac La Nonne, and "take-overs" by Pseudanabaena catenata.

Year	Dates Blooms Collected	Aphanizomenon Number of Isolates Initial	After 1 wk.	After 4 wks.	Pseudanabaena Take-overs After 4 wks.
1980	Sept 17	24	6	0	6
1981	July 27	36	14	4	2
	Aug 27	37	20	0	19
	Sept 27	45	13	2	4
	Sept 27	12	12	0	12
	Oct 6	24	24	0	24
	Totals[1]	178	89	6	67

[1]Isolates not accounted for were discarded because of lysis, contaminating green algae or protozoa.

The "take-over" isolates appeared yellow-green, green-brown or brown-purplish in suspension (Plate 1) and after lyophilization (Plate 2) and exhibited poor to good growth. Bacterial contaminants ranged from trace to 5+ (ca. 25 x 10^7 per ml.) and included many different species. The flexuous filaments consisted predominately of somewhat irregular chains of subcylindric cells without heterocysts, akinetes or obvious sheaths. Occasional jerky bending and straightening or waving movements were seen in a few mounts. The cells were 1.4 to 2.5μm in diameter x 1.3 to 2.5 or 1.9 to 10.0, occasionally to 34μm long, with or without some irregular (bulged, curved, S-shaped, branched or coccoid) cells and misshaped tip cells. There were filaments with cells that were without and filaments with cells that had faint to prominent polar and/or sub-polar gas vacuoles, and/or faint to prominent random gas vacuoles (Appendix 2).

The absence of prominent polar gas vacuoles and the pronounced purple colour of the freeze-dried suspensions of "take-over" culture A81-15(B1) and filament isolates from it initially caused the cyanobacterium to be referred to as *Pseudanabaena sp.* (Gorham *et al.*1982). However, comparison of strain A81-15(B1)-g (Plate 3) with *Pseudanabaena catenata* Lauterb. CCAP 1464/1 and *Pseudanabaena brunea* (auct.?) CCAP 1464/2 (Plates 4 and 5) and the other "take-over" isolates made it seem that all were forms of *P. catenata* Lauterb. that vary in cell diameter, cell length, shape of tip cells, gas vacuoles, pigments and motility.

Plate 1

To illustrate colour differences between a culture of *Aphanizomenon flos-aquae* A81-31(C5) (left 2-litre flask) and a "take-over" culture of *Pseudanabaena catenata* (right 2-litre flask).

Plate 2

Freeze-dried powders illustrating the colour differences between four strains of *Pseudanabaena catenata* "take-over" cultures (left) and an *Aphanizomenon flos-aquae* culture (far right).

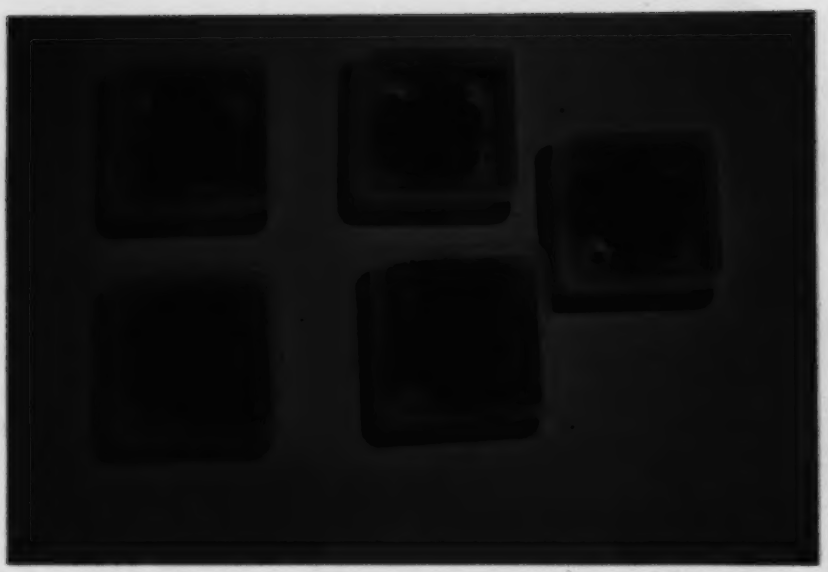


Plate 3

Non-toxic clone of Pseudanabaena catenata Lauterb. A81-(B1)-g (a brown-purple form) Isolator: P.R. Gotham.

a) Phase Contrast 1000x

Fragments and one long flexuous trichome consisting of chains of regular subcylindric cells of variable length. No gas vacuoles are clearly discernable.

b) Nomarski 2560x

Two trichomes that show a difference in diameter. Random gas vacuoles appear as shallow depressions. Prominent polar gas vacuoles appear as deep depressions.

Plate 4

Pseudanabaena catenata Lauterb. CCAP 1464/1 (a green form) Isolator: E.G. Pringsheim.

a) Phase Contrast 1000x

Short flexuous trichomes consisting of chains of regular subcylindric cells of variable length. Prominent terminal and random gas vacuoles are visible in certain trichomes appearing as bright spots. Faint polar gas vacuoles account for apparent spacing of cells.

b) Nomarski 2560x

Two trichomes with terminal gas vacuoles which appear as bright hemispheric spots at bottom ends. Bright spots in and between cells are random and/or polar gas vacuoles.

Plate 5

Pseudanabaena catenata Lauterb. CCAP 1464/1 (a green form) Isolator: E.G. Pringsheim.

a) Nomarski 2560x

Single trichome in mount sealed with nail polish for 27 hours. Prominent terminal gas-vacuoles at bottom end of trichome. All other cells show only random gas vacuoles.

b) Nomarski 2560x

Single trichome in mount sealed with nail polish for 27 hours showing a pronounced bend. One cell has a large gas vacuole that occupies virtually the entire cell. Sub polar and random gas vacuoles appear as bumps or depressions.

Rippka *et al.*(1979) have suggested that a major diagnostic feature of this species is the presence of prominant polar gas vacuoles in each individual cell of a filament. However, Bourrelly and Caute (1975) changed the name of strain 391 of the Algotheque of the Museum National d'Histoire Naturelle, Paris from *P. catenata* Lauterb. to *P. galeata* Bocher because *P. catenata* is supposed to lack pseudovacuoles! In aqueous mounts of *P. catenata*, CCAP 1464/1 and *P. brunea* CCAP1464/2 that were sealed with nail polish for 2h, it was found that the prominent polar gas vacuoles became faint and disappeared while faint to prominent random gas vacuoles appeared and remained stable for 24 to 27h (Plates 6 and 7). Therefore, prominant polar gas vacuoles are not reliable as a diagnostic feature for this species since their presence seems to be subject to change depending on environmental conditions. Because of the range of variation observed in the different cultures and the prevailing confusion about the taxonomy of *Pseudanabaena*, it was decided to be conservative and refer to all the "take-over" cultures (and isolates derived from them) as *P. catenata* Lauterb.

Plate 6

Pseudanabaena brunea (auct?) CCAP 1464/2 (a green-brown form) Isolator: E.G. Pringsheim.

a) Phase Contrast 1000x

Several flexuous trichomes consisting of chains of regular subcylindric cells of variable length. Prominent polar and terminal gas vacuoles can be seen in all trichomes as bright spots.

b) Nomarski 1560x

Four trichomes with terminal and polar gas vacuoles which appear as deep depressions. Random gas vacuoles appear as shallow depressions.

Plate 7

Pseudanabaena brunea (auct?) CCAP 1464/2 (a green-brown form) Isolator: E.G. Pringsheim.

Nomarski 2560x

Two trichomes in mount sealed with nail polish for 27 hours. Prominent terminal gas vacuoles remain, but polar gas vacuoles have disappeared and have been replaced by many random gas vacuoles, which appear as bumps and depressions.

3.3 Media and culture conditions for cyanobacteria

Purified distilled water was used throughout. It was prepared by passage through a Barnstead organic filter followed by a cation removal cartridge.

The media used initially for culturing *Aph. flos-aquae*, *Anab. flos-aquae*, *Anab. sub-cylindrica* and *P. catenata* were ASM-1 and ASM-2 (Carmichael and Gorham 1974). These media were found unsuitable for optimum growth of *Aph. flos-aquae* and *P. catenata* and were replaced by P-1 and P-2 media (Table 2). P-1 medium is a modified form of ASM-1. Buffering is increased by the replacement of Na_2HPO_4 by an equimolar amount of $Na_3PO_4.12H_2O$ (pH 8.5). The exclusion of $ZnCl_2$ and the reduction of $NaNO_3$ to $^1/_4$ strength are the other modifications. P-2 medium consists of P-1 medium plus the same mixture of vitamins and peptones that are used in ASM-2 medium. P-3 medium consists of P-2 media plus a supplement of the yeast extract and dextrose components of Difco Plate Count Agar at $^1/_{100}$ strength.

Agar media were prepared by using washed gels of 1.5% Oxoid or 2% Difco Bacto agars combined 50:50 with the respective autoclaved double-strength medium (pH 8.5) after UV-sterilization and minimal heating of the agar to 70°C in a microwave oven, as described by Allen and Gorham (1981). For more reliable growth, the agar was washed with 2mM $NaHCO_3$ (pH 8.5) rather than purified water. The Marine Colloids Sea Plaque agar and Marine Colloids Low-Temperature-Gelling Agarose that were tried were

obtained from Mandel Scientific Company, Rockwood, Ontario.

Isolations, washings and transfers were made in a laminar-flow hood. Washed filaments were cultured in screw-capped tubes, Delong flasks or Petri dishes at a constant 21°C in continuous cool white fluorescent light. The photosynthetically active radiation (P.A.R.) 400-700nm of this light was 40μE m^{-2} s^{-1}, as measured with a Lambda L1-185 quantum sensor and meter.

Table 2. Composition of P-1 basal medium and modifications used.

P-1 medium	mg/l	P-2 supplement	mg/l
$NaNO_3$	42.5	Thiamine	0.20
K_2HPO_4	17.4	Pyridoxine	0.16
$Na_3PO_4.12H_2O$	38	Calcium pantothenate	0.08
$MgCl_2$	19.02	Riboflavin	0.02
$MgSO_4$	24.08	Biotin	0.01
$CaCl_2$	22.2	Folic acid	0.002
$FeCl_3$	0.65	Cyanocobalamin	0.001
HBO_3	2.47	Sodium caseinate (Difco)	10.0
$MnCl_2$	0.87	Tryptone (Difco)	10.0
$CoCl_2$	0.01	Proteose peptone (Difco)	10.0
$CuCl_2$	0.0001		
Na_2EDTA	6.64		

P-2 medium consists of P-1 medium plus the P-2 supplement.

P-3 medium consists of P-2 medium + a supplement of yeast extract (Difco) 25.0 mg and Dextrose (Difco) 10 mg per 1.

P-3 soil extract medium consists of P-3 medium + 2 to 10 ml/1 soil extract.

P-1, P-2, P-3 and P-3 Soil Extract Agars consist of the respective double-strength media added to equivolumes of 2% Bacto washed agar (pH 8.2 to 8.5).

3.4 Isolation and culture of bacteria

The major bacterial contaminants of an actively growing 21-day-old sub-culture of A81-15(B1)-g were isolated on Bacto Plate Count Agar. A dilution factor of 10^5 produced 148 colonies on the agar surface indicating a total bacterial count of 1.48×10^7 cells/ml of culture. Five predominant bacterial species were isolated by repeated restreakings and mass cultured in 2l of Nutrient Broth for 48h at 28°C on a gyrorotary shaker. The bacterial suspensions were harvested using a Sharples centrifuge and freeze-dried. Freeze-dried cells were resuspended and intraperitoneally injected into male mice to test for any possible toxic action at a dosage level >1500mg kg^{1} body weight.

3.5 Methods of toxicity-testing

Cultures were freeze-dried and tested for toxicity by preparing thick suspensions (64mg/2.0ml) which were injected intraperitoneally into 20-g ALAS strain mice (male), using two per dosage level. Disposable Plastipak syringes and needles were used throughout. The mice were examined hourly for 8-12 hours and autopsied to ascertain if any liver damage or other abnormalities were visible.

3.6 Equipment and materials for purification

For Kraus's (1969,1976) ^{60}Co method an Atomic Energy of Canada Ltd. Gammacell with an output of 73.2krad h^{-1} was used.

For Parker's (1982) Na_2S drop method ASM-1/washed Bacto agar containing 2mM Na_2SO_3 was used. The 2.0M Na_2S was dissolved in air and used immediately instead of being dissolved under nitrogen and stored for use.

For Vaara and Vaara's (1979) antibiotic enrichment method P-2 medium was used rather than TYG broth. The antibiotics ampicillin (Ayerst Labs. Montreal, Canada) and D-cycloserine (Sigma Chemical Co, St. Louis) were tested at concentrations of 1, 1.5 and 2.0mg/ml.

For Carmichael and Gorham's (1974) method, phenol was used at concentrations of 0.1, 0.2, and 0.3% w/v.

For washing, the second method described by Gorham *et al.* (1982) was used. The washing apparatus is pictured in Plates 9 and 10. Depending on the viscosity of the washing medium, Millipore and, later, Nucleopore membranes having pore sizes of 1.2, 5.0 and 8.0 µm were used.

The following high-molecular-weight colloids were obtained from Sigma Chemical Co. and were tested as cell envelope protectants during washing: Polyvinylpyrrolidinone commonly called Polyvinylpyrrolidone or PVP (MW 10,000, 40,000, 40,000T, 360,000), dextran (MW 10,300, 40,000, 81,600, 252,000, 510,000), Ficoll (MW 70,000, 400,000, 400,000DL), inulin (MW 7200, 7360) and gelatin.

Plate 8

Washing apparatus used for the reduction of bacterial flora, and the recovery of *Pseudanabaena catenata* from a mixed cyanobacterial/bacterial culture.

Plate 9

Recovery of filaments of Pseudanabaena catenata after washing with P-1 medium and/or dextran (81,600 1%).

3.7 Isolation and testing of functionally axenic strains

Colonies of cyanobacteria from single filaments in pour-plates that showed no obvious bacterial association were isolated by coring with a Pasteur pipette or a needle having a 1.0μm diameter tip (Minuten insect pin). Cultures exhibiting the lowest bacterial count were washed with dextran 81,600, overlay-plated in P-3 washed agar pH 7.0, 7.5, 8.0, 8.5, and 9.0, and incubated under constant light at 21, 28 and 35°C. Each plate was examined microscopically (x400) over a period of 21d to check for contamination after which time further isolations were made. These isolates were transferred to successively larger volumes of P-3 medium up to, and including, 250ml.

Contamination of the isolates was tested after one month by adding 0.2ml of actively growing culture to washed agar plates of P-3 Agar, Plate Count Agar, Trypticase Soy Broth Agar and Nutrient Agar and incubating at 21°C for 10 days. Those plates from cultures that appeared to be functionally axenic were sealed with Parafilm and incubated under constant light at 21°C for three months to provide an opportunity for any latent bacterial contaminants to become visible.

4. Results and conclusions

4.1 Comparison of commercial agars for culturing planktonic cyanobacteria

A number of commercial washed agars were used to gel ASM-1 medium by a modification of Allen and Gorham's (1981) method. Growth of *P. catenata* and other species of planktonic cyanobacteria on the different agars was compared to see which was most suitable for further use (Table 3).

Sea Plaque agar proved to be the least effective. All species and strains exhibited clipping and, or, lysis when pour-plated into, or placed upon its surface. Low-Temperature-Gelling Agarose, despite the obvious benefit of permitting plating at a much lower temperature than the other agars, provided suitable growth for only *An. sub-cylindrica*. *P. catenata* showed signs of very slow growth, but clipping and stasis was also prevalent.

Both Oxoid No.1 and Difco Bacto agar were found suitable for most species after washing and raising their pH to about 8.5. Difco Bacto agar was chosen for further use as *P. catenata* grew best with it.

Table 3. Growth of P. catenata and three other species of planktonic cyanobacteria on four commercial agars (1%) washed with 2mM$NaHCO_3$, pH8.5.

Species	Sea Plaque	Low Temperature Gelling Agarose	Difco Bacto	Oxoid No. 1
P. catenata	tr(c, 1)*	tr(c, 1)	5+	5+
An. sub-cylindrica	3+	tr(c, 1)	5+	5+
Aph. flos-agriae	--		5+	5+
M. aeruginosa	--		5+	5+

*Growth rate estimated as tr = trace, 3+ = medium, 5+ = maximum;

c = clipping, lysis of one or a few adjacent cells in a filament; l = lysis, disintegration of all or nearly all cells of a filament leaving only ghosts

4.2 Toxicity of P. catenata "take-over" cultures

Intraperitoneal injection of two of the 67 "take-over" cultures into mice caused violent convulsions and a loss of sensitivity to touch by the eyes and ears. A paling of the outer margins of each liver lobe was seen after the animals were sacrificed and autopsied.

To check whether the convulsant activity might be of bacterial origin, the five major bacterial contaminants of a 21-day-old sub-culture of A81-15(B1) were isolated, mass-cultured, and suspensions of freeze-dried cells injected intraperitoneally into mice at a dosage level of >1500mg kg^{1} body weight. None of the five bacterial isolates produced convulsions such as were witnessed with suspensions of freeze-dried A81-15(B1) culture. They only exhibited signs of slow death over a 24-to 36-h period characteristic of bacterial endotoxic poisoning.

4.3 Methods of isolation and purification

Over the years, many methods based on selective destruction of contaminants have been published, but, generally speaking, none have proved to be as satisfactory as their authors claimed, especially for purifiying planktonic cyanobacteria. Several of the selective methods were tried before efforts were concentrated on improving a method of physical separation.

4.3.1 60-Cobalt Gamma Radiation

Actively growing cultures of *An. flos-aquae* NRC 44-1, *Aph. flos-aquae* A81-31(C5) and *P. catenata* A81-15(B1) were used to test Kraus's purification method in two sets of experiments. Cultures were treated in ASM-2 or P-2 medium in screw-capped tubes to a dosage range of 100 to 400krad in 10krad intervals, with and without a 24-h dark post-irradiation treatment. The treated cultures were incubated in both ASM-2 or P-2 media and agar pour-plates and, as streaks, on Difco Plate-Count Agar. The cultures were examined on a weekly basis, with observations lasting from 4 to 6 weeks.

The results indicated that gamma rays could not be used as a selective antibacterial agent. At all dosage levels tested,including the lowest, the cyanobacteria proved to be more susceptible to radiation damage than the bacteria. The three test species of cyanobacteria showed stasis or lysis whilst the contaminating bacteria survived and grew (Table 4).

Table 4. Attempts to purify cultures or reduce their bacterial populations by the ^{60}Co gamma radiation method of Kraus.

Cultures[1]	No. Expts.	Dosage, krad	Dark 24 h	Growth at Lowest Dosage[2] pH	cyano.	bact.
An. NRC 44-1	2	250-400	no	8.2	0[3]	3+ to 5+
An. NRC 44-1	2	250-400	yes	8.2	0	3+ to 5+
Aph. A81-31 (C5)	2	200-400	no	6.8	0	1+
Aph. A81-31 (C5)	2	200-400	no	8.5	0	5+
Pseu. A81-15 (B1)	2	100-200	no	8.5	0	5+
Pseu. A81-15 (B1)	2	100-200	yes	8.5	0	5+

[1] 21-day, bacteria 5+, except Aphanizomenon 2-day, bacteria 3+.

[2] In both ASM-2 or P-2 liquid media and agar pour plates and on Difco Plate Count agar; observed weekly for 4-6 weeks. 5+ = ca. 25 x 10^7 bacteria per ml.

[3] 0 = statis and/or lysis in days or weeks.

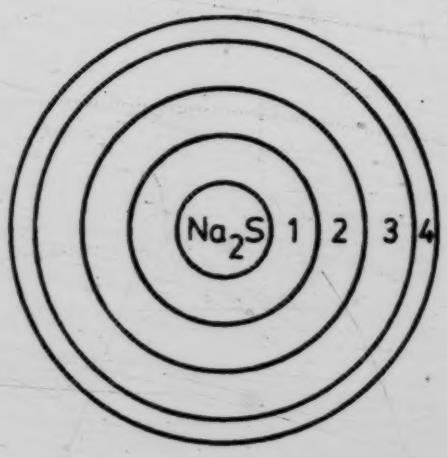
4.3.2 Parker's Sulphide Method

Parker's sulphide method was tried on three test species *Aph. flos-aquae* A81-31(C5), *An. sub-cylindrica* A78-1 and *P. catenata* A81-15(B1)-g. The complex gradients of pH and of sulphide and sulphite concentrations caused a gradation in numbers and colony size of the bacteria from the centre to the edge of the pour-plate but failed to eliminate them completely from the centre. *P. catenata* and *Anabaena* showed gradations in growth response but they were far more susceptible to damage by the higher concentrations than the bacteria. *Aphanizomenon* was the most susceptible to the gradations in pH, sulphide and sulphite concentrations. It lysed over the entire plate (Gorham *et al*.1982) (Fig 1).

Figure 1. Trials of Parker's sulphide selection method for purifying a clone of *Anabaena sub-cylindrica* A78-1, a take-over culture of *Pseudanabaena catenata* Lauterb A81-15(B1)-g and a colony isolate of *Aphanizomenon flos-aquae* A81-31(C5).

The sulphide and pH gradients decreased from the zone labelled Na,S to the zone labelled 5. Growth of bacteria and cyanobacteria is designated by 0 = none, st = stasis, tr = trace, 1+ to 3+ = some to many bacteria.

Parker's Sulfide Method



	Na_2S	1	2	3	4
Bacteria	tr	1+	2+	3+	3+
An. s. A78-1 or Pseu. A81-15	0	st	tr	2+	3+
Aph. A81-31	0	0	0	0	0

4.3.3 Antibiotics and Antiseptics

The antibiotic enrichment method used by Vaara and Vaara (1979) for the purification of their strains of *Pseudanabaena* was tried with strain *P. catenata* A81-15(B1)-g. The culture was dark-starved in P-2 medium for 48 to 96h after which the antibiotics ampicillin or D-cycloserine were added at concentrations of 1.0, 1.5 or 2.0mg/ml. The cultures were returned to the dark for a further 24 to 96h. The antibiotics were then removed by the gravity-washing procedure described in the Materials and Methods Section.

Filaments were still present after a 24-h incubation with 1.0 mg/ml D-cycloserine but had disappeared in the 1.5 mg/ml, 2.0 mg/ml tubes and in all the ampicillin-enriched tubes. Both antibiotics caused *P. catenata* to lyse completely when incubated in the dark for 48 or 96h. Upon washing, to remove all traces of the D-cycloserine from the 1.0 mg/ml, 24-h dark treatment, the remaining filaments of *P. catenata* disappeared. Filaments could not be found on the filter membrane or in the solution above indicating lysis had occurred during the washing procedure. Cycloserine was found to reduce the bacterial population to a far greater extent than ampicillin, but at the concentrations tested neither exhibited adequate selectivity for the heterotrophic bacteria in preference to the obligate photoautotrophic cyanobacteria.

The phenol enrichment method of Carmichael and Gorham (1974) was also tried with strain *P. catenata* A81-15(B1)-g. It was dark-starved in P-2 medium for 24 to 96h, after which time phenol was added at concentrations of 0.1, 0.2 or 0.3% w/v. The cultures were then returned to the dark for an additional 3 to 6 hours. Samples were removed at 30-min intervals, gravity- instead of suction-washed and pour-plated in P-2 washed agar. The plates were sealed with Parafilm and incubated for one month.

The results were totally unsatisfactory. The lowest concentration of 0.1% phenol caused complete lysis of the *P. catenata* cells within 30min of application. The use of this method with other planktonic strains of cyanobacteria has shown that it is not always reliable (Gorham, personal communication).

4.3.4 High-Molecular-Weight Colloids as Membrane Protectants

Attempts were made to improve the recovery of viable filaments from the washing procedure described by Gorham *et al.*(1982), by adding various concentrations of different kinds of inert, high-molecular-weight colloids to the P-1 washing medium. An initial screening of PVP, dextran, Ficoll, gelatin and inulin, was performed with *P. catenata* A81-15(B1)-g.

Aliquots of 0.5ml (150 to 175 filaments) were gravity-filtered with 5x10ml of each concentration of each kind and type of colloid. The efficiency of the washing

procedure and the protective properties of the colloidal suspensions were assessed by:

(1) counting the number of filaments present in 0.5ml of the initial inoculum;

(2) recording the number of filaments present directly after pour-plating;

(3) scoring the number of filaments and the number of bacteria exhibiting growth in the following week.

The results of the screening process (Table 5) indicated that seven of the colloid treatments promoted favourable filament recovery (20% recovery or greater). These treatments were examined more intensively by increasing the number of washes from 5 to 10. Under these conditions only two colloid treatments promoted substantial filament recovery. These were dextran 81,600 at 1%, and Ficoll 400DL at 5%.

Some problems were encountered with the various colloid series. In particular, the very high viscosities associated with solutions of gelatin made filtration impossible at concentrations greater than 1%. Similarly, the highest-molecular-weight members of both the PVP and dextran series could be filtered only through a 5μm porosity filter over a time period of 90 minutes.

Table 5. The use of inert, high-molecular-weight colloids as a washing media for Pseudanabaena catenata Lauterb. A81-15(B1)-g.

Colloid	M.w. x 10^3	.125	0.5	1	2	3	4	5
PVP(a)	10	-	-	-	-	-	-	-
	40	-	-	<20*	-	-	-	-
	40T	-	-	-	<25*	-	-	-
	360	-	<5*	<20*	<5*	-	-	-
FICOLL(a)	70	-	-	-	-	-	-	-
	400	-	-	-	-	-	<5*	<10*
	400D L	-	-	-	-	<5*	<15*	<30*
DEXTRAN(a)	10	-	-	-	-	-	-	-
	40	-	-	-	-	-	-	-
	82	-	<40*	<75*	<35*	-	-	-
	252	-	<10*	-	-	-	-	-
	510	-	-	-	-	-	-	-
INULIN(a)	7.1	-	-	-	-	-	-	-
	7.3	-	-	-	-	-	-	-

(a) Filaments of P. catenata were washed in 5 x 10 ml of each particular molecular weight and concentration in P-1 medium.

*Recovery rates are based upon the number of filaments of P. catenata showing signs of growth (with no evidence of clipping) after 24 hours in P-2 washed agar pour-plates.

Dextran 81,600 at 1% in P-1 medium was finally selected as the optimal washing solution. Growth was evident in each filament that emerged from the washing process within 24h. Viable filaments of *P.catenata* could be recovered even after 15x10ml washes. Ficoll 400DL at 5% was effective in protecting 30% of the filaments. The remaining filaments showed signs of clipping, and in some cases, lysis, within 24h of plating. A closer examination of dextran 81,600 revealed that filament recovery and viability could only be guaranteed between closely delineated concentrations. Recovery peaked between 0.8 and 1.2% and dropped rapidly below 0.5% and above 1.5%.

It was initially hypothesized that recovery rates with the various colloids might be associated with increased osmotic stresses resulting in membrane damage. However, a closer examination of the osmolarities of the individual colloidal suspensions indicated that the osmotic stresses placed upon individual cells would be almost negligible at the concentrations being used. Further, when colloids whose initial osmolarities differed from that of dextran 81,600 at 1% were adjusted to a corresponding value no increased viability was observed in the filaments of *P. catenata*.

4.4 Mode of action of dextran 81,600

A series of Scanning Electron Micrographs (SEM) were taken of *P. catenata* A81-15(B1)-g fixed by freeze-substitution after washing in either purified

distilled water, P-1 medium or dextran. Specimens were fixed at time zero, 30m and 3h after washing.

The micrographs of *P. catenata* washed in water (Plates 10,11) indicate what appears to be a deposition of some salts or other substances on or a peeling of patches of the outermost wall layer. Plates 12 and 13 illustrate filaments of *P. catenata* after a wash in P-1 growth medium. No discernible differences were visible in the cellular morphology over the entire time course. The process of freeze-substitution caused the filaments to collapse somewhat but there was no evidence of the loss of an outer wall layer or deposition of substance as seen with the wash in distilled water.

Plates 14 to 17 illustrate filaments of *P. catenata* washed in a 1% solution of dextran 81,600. The filaments showed no signs of clipping or loss of an extra-membranous covering. Rather, the micrographs provided evidence of what can only be a dextran deposition upon each cell. The thickness of this coat increased as the time course ran to its limit. After 3h, the filaments were so completely covered with dextran that it obscured the outlines of the cells.

Although the filaments of *P. catenata* would not have remained for 3h in dextran during the actual washing process the deposition of a light coating can be detected at time zero. The indications from the electron micrographs are, therefore, that dextran becomes attached to the walls of the

individual cells.

Plate 10

Pseudanabaena catenata (Lauterb) A81-15(B1)-g

Glutaraldehyde - OsO_4 fixation

S.E.M. 9300 x Time 0

Trichomes were washed in 5 ml of sterilized purified water. Note the deposition on/or the peeling of patches of the outer envelope of the cells.

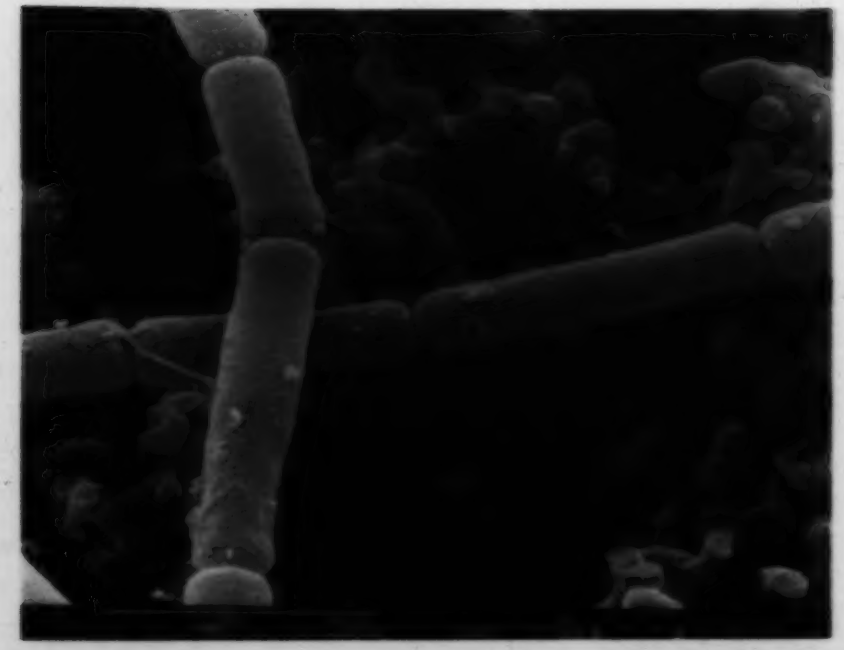
Plate 11

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Glutaraldehyde - OsO_4 fixation

S.E.M. 22,000 x Time 0

Trichomes were washed in 5 ml of sterile purified water. Arrow = area of deposition on/or the peeling of patches of the outer envelope of the cells.



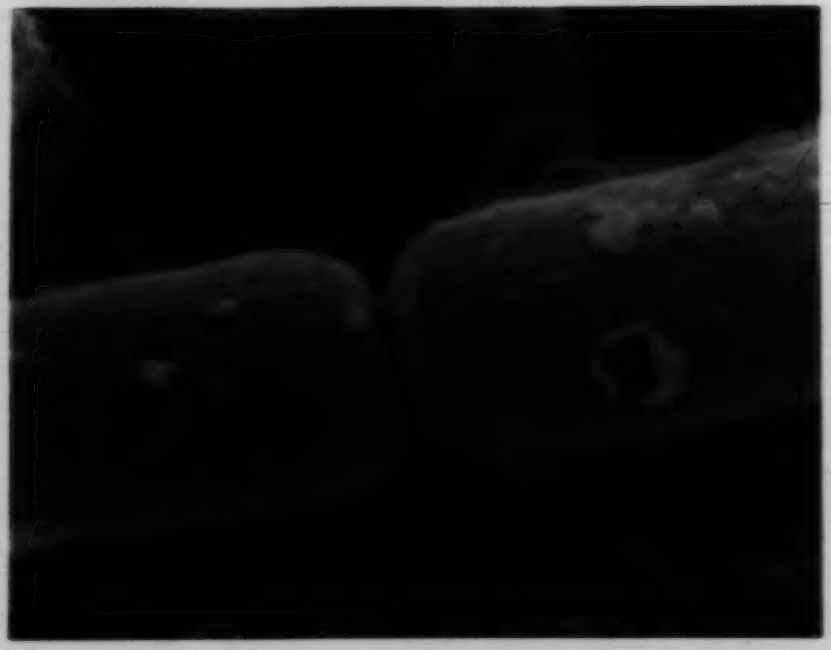


Plate 12

Pseudanabaena catenata Lauterb. A81-15(b1)-g

Freeze - substitution fixation

S.E.M. 15,600 x Time 0

Trichomes were washed in 5 x 10 ml of sterile P-1 medium. There is no deposition on/or peeling of patches of the outer envelope of the cells that show regions of collapse due to freeze-drying.

Plate 13

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Freeze - substitution fixation

S.E.M. 15,400 x Time 3 hours

Trichomes were washed in 5 x 10 ml of sterile P-1 medium (and allowed to sit in P-1 for 3h). Freeze-substitution has caused the cells to collapse, but no evidence of rupturing is visible.

Plate 14

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Freeze - substitution fixation

S.E.M. 5,200 x Time 0

Trichomes were washed in 5 x 10 ml of 1% dextran (81,600). Some trichomes are slightly obscured and lying across an 8 ^{u}m pore, the bottom portion of which is covered with a layer of a substance, presumably dextran.

Plate 15

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Freeze - substitution fixation

S.E.M. 11,400 x Time 30 minutes

The trichomes were washed in 5 x 10 ml of 1% dextran (81,600) and then allowed to sit in the medium for 30 minutes. The trichomes are coated with, and show strands of dextran attaching them to the filter. Drying has caused gaps to appear between some of the cells.

Plate 16

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Freeze - substitution fixation

S.E.M. 15,000 x Time 30 minutes

The trichomes were washed in 5 x 10 ml of 1% dextran (81,600) and allowed to sit in the medium for 30 minutes. The trichomes are obviously coated by a layer of dextran which has dried and torn causing gaps between the cells during the process of freeze substitution.

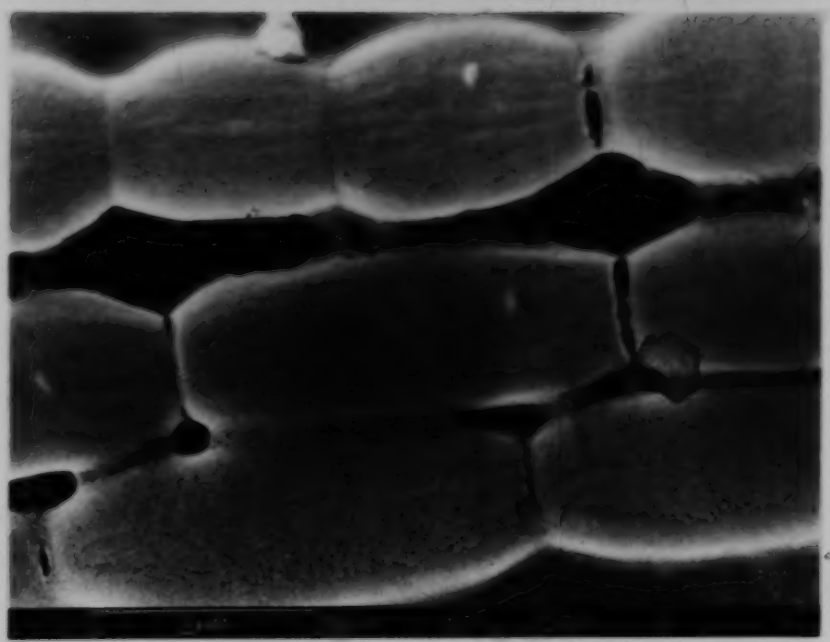


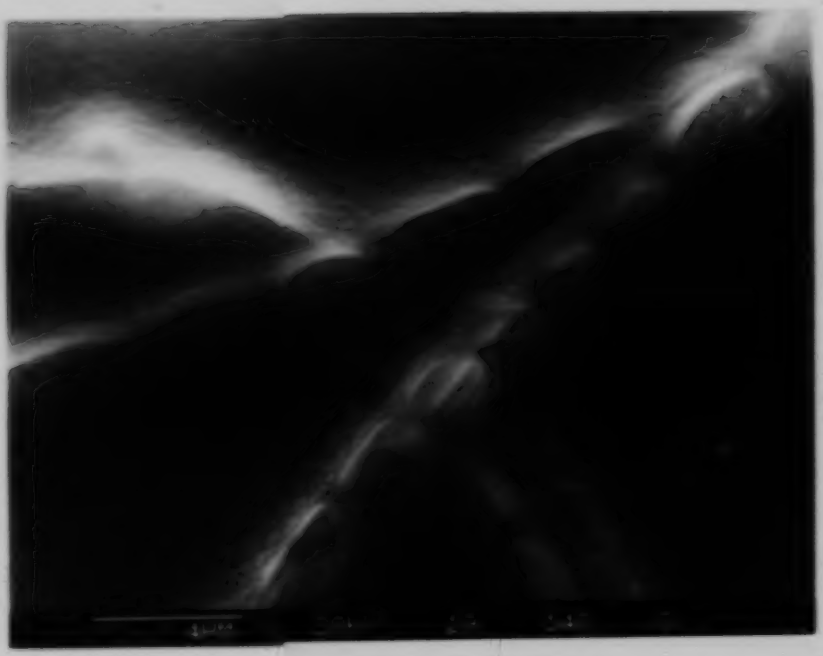
Plate 17

Pseudanabaena catenata Lauterb. A81-15(B1)-g

Freeze - substitution fixation

S.E.M. 4,700 x Time 3 hours

The trichomes were washed in 5 x 10 ml 1% dextran (81,600) and allowed to sit in the medium for 3 hours. The individuality of the filaments is completely obscured by the deposition of dextran.



4.5 Antibiotic and Antiseptic Agents with Dextran

Since *P. catenata* is susceptible to washing damage, the failure of the antibiotic and antiseptic enrichment methods may have been caused, in part, by the need for a protective colloid during the dark-treatment and/or washing steps involved.

The effects of adding 1% dextran 81,600 during a 48-h dark incubation period with 1.0 or 2.0mg/ml D-cycloserine, or with 0.1% phenol (w/v) for 1, 3 and 5h were studied with *P. catenata* A81-15(B1)-g using a 0.5ml/10ml dilution in P-1 medium of log-phase culture. Filaments were examined microscopically for clipping and lysis after the dark treatments, then given 5x10ml washes in 1% dextran 81,600 and pour-plated in P-2 washed agar. The plates were examined microscopically immediately after pouring and daily thereafter for 7 days.

The presence of dextran 81,600 in cultures of *P. catenata* during dark treatment with D-cycloserine or phenol or in the washing step that followed provided no improvement in survival compared with controls in which they were absent.

4.6 Recovery of axenic strains of P. catenata

To better estimate the efficiency of the washing process an overlay-plating method was adopted. Instead of the final 0.5ml of washed filaments being diluted to 10ml, combined with 10ml P-2 washed agar medium (P-2A) and poured,

it was added to 1ml of P-2A (pH-adjusted) and poured as an overlay on P-2A. In the overlay gel, individual bacteria and cyanobacterial, filaments were spatially separated and fixed in a thin plane that corresponded with the depth of field of the inverted microscope used to examine them at a magnification of 200x. Viable counts could thus be obtained more rapidly and reproducibly than before. Overlay plates of *P. catenata* A81-15(B1)-g given a single wash with dextran 81,600 were made with P-2A at pH 7.0, 7.5, 8.0, 8,5, and 9.0 and incubated in constant light at 21, 28 and 35°C for seven days. Microscopic inspection (x200) showed that at 35°C both the bacteria and cyanobacteria were absent at all five pH values. At 28°C, bacterial contaminants were found in greatest numbers at pH 7.5 and 8.0 whilst *P. catenata* was clipped and in stasis at all pH's. At 21°C good growth of bacteria and *P. catenata* occurred at pH 7.5, 8.0 and 8.5. There were distinct changes in the growth habitat and colony appearance of the bacteria from those incubated at 28°C.

Comparison of 10x10ml and 15x10ml washings with dextran 81,600 provided very little difference in terms of the total numbers of bacteria still remaining. *P. catenata* A81-15(B1)-g however, showed a marked decline in the percentage of viable filaments that could be recovered as the number of washes increased from 10 to 15. The large-scale reduction in the bacterial population achieved by 10 washes resulted in *P. catenata* at 21°C growing well at all pH values from 7.0 to 9.0. Optimal growth now occurred

at pH 7.5 rather than pH 8.5 where it had occurred prior to washing. Under these conditions, using overlay plates on P-2A, the number of clearly discernible bacterial colonies ranged between 8 and 12 per 90-mm plate after 7 days' incubation.

By combining (1)the optimum washing procedure, (2)spatial separation by overlay plating, (3)single filament re-isolation, (4)bacteriological testing with different media, pH;s and incubation temperatures and repeating each step (Fig 2), functionally axenic strains of *P. catenata* were produced

After washing with dextran 81,600 and overlay plating on P-2A, 27 colonies (from single filaments), which appeared by microscopic examination at 200x to be adequately separated from any bacterial colony, were removed with a Minuten insect pin and cultured in P-3 medium at 21°C for 2wk (Fig 2). An aliquot (0.2ml) of each isolate was distributed upon the surface of Plate Count Agar (PCA) and P-2A and incubated under constant light at 21 and 28°C for 2wk. Isolates on P-2A that showed no obvious contaminants on both test media were cored and the isolates re-grown in P-1 medium.

Of the 27 isolates, 10, which appeared to be free of obvious contaminants, were tested at two temperatures on three bacteriological media prepared with washed agar to check again for contaminants. A sample (0.2ml) of each culture was spread over the surface of plates of washed

Nutrient Agar (NA), P-3 soil extract washed agar (P-3ASE), and Trypticase Soy Broth washed agar (TSBA)and incubated at 21 and 28°C for 2wk.

Of the 10 isolates that appeared to be bacteria-free, four showed no obvious contaminants on any of the three test media. Six, however, showed contamination on P-3ASE. The four apparently uncontaminated strains (A81-15(B1)-g-14,-19,-22, and-25) showed no contaminants when re-tested on P-3ASE at 21°C for 4wk.

Strain A81-15 (B1)-g-22 (now 6wk old) was diluted and overlay-plated in P-3A. After 10 days, 20 cores were taken and placed in P-3 medium and grown at 21°C under constant light. Eleven of these isolates showed signs of growth 1wk later and were tested on plates of NA, P-3ASE and TSBA that were incubated at 21 and 28°C for 4wk.

Of the 11 reisolated strains of -g-22-, nine (-g-22-b, -c, -f, -h, -k, -l, -r and -s) showed no obvious bacterial contamination by microscopic inspection at 200x one month later and were considered to be functionally axenic.

Figure 2. Diagram of the steps involved in the improved washing method for obtaining functionally axenic strains of P. catenata A81-15(B1)-g. PCA = Plate Count Agar; NA = Nutrient Agar, P-3ASE = P-3 Washed Agar and Soil Extract. TSBA= Trypticase Soya Broth P-2A = P-2 washed agar. * a apparently uncontaminated.

Bacteriological Test Media and Conditions		Dextran-washed Clone A81-15(B1)-g colony isolation
PCA P-2A	2 wk 21°, 28°C	27 re-clones (-g-1 to 27) 20*
NA P-3ASE TSBA	2 wk 21°C, 28°C	10*
P-3ASE	4 wk 21°C	4* (-g-14,-19,-22,-25)
		Overlay plated A81-15(B1)-g-22
		20 re-clones (-g-22-a-test)
NA P-3ASE TSBA	4 wk 21°C, 28°C	11 grew
		9* (-g-22-b,-c,-f,-h,-k,-l,-p,-r,-s)

5. Discussion

The search for a type-*c* toxic strain of *Aphanizomenon flos-aquae* from Lac La Nonne, Alberta, proved to be unsuccessful. The search, however, has led to several new findings about growth requirements and membrane integrity of *Pseudanabaena catenata* and other cyanobacteria and better ways to obtain and test for purity of functionally axenic clones.

Although only a minor constituent of the blooms that were dominated by *Aph. flos-aquae*, strains of *P. catenata* were very aggressive in culture, "taking-over" 67 of 73 isolates in a relatively short period of time. Since the occurrence of the "take-overs", *Aphanizomenon* colonies or filaments have not re-emerged in culture, indicating that *P. catenata* behaved very much like the types of bacteria that cause lysis and exclusion of planktonic cyanobacteria in both liquid and agar media.

Mason *et al.* (1982), have recently described cyanobacterin, from the cyanobacterium *Scytonema hofmanni*, which acts as an anticyanobacterial agent against a range of alternate species. The discovery of such an agent suggests the possibility that similar compound(s) may have contributed to the "take-over" of cultures by *P. catenata*.

Two "take-over" cultures were found to exhibit convulsant activity. However, these were lost, perhaps by successional changes, whilst unsuccessful attempts were being made to isolate a toxic strain(s).

In lacustrine conditions, the appearance and disappearance of toxic blooms on a day-to-day basis is well documented (Carmichael and Gorham 1981). Bloom toxicity is correlated with localized or generalized dominance of one or more toxic strains. The decline or loss of toxicity is linked to dispersion and mixing. It may also involve abolition or suppression of the correct genetic coding required to maintain a state of toxicity (Hauman 1981, Hauman *et al*.1982).

The possibility that convulsant activity was caused by a bacterial endotoxin was investigated. Freeze-dried extracts of the five major Gram-negative contaminants from the two toxic "take-over" cultures were intraperitoneally injected into mice. They exhibited signs of slow death characteristic of bacterial endotoxic poisoning. Since it is unlikely that the dominant bacterial species in this "take-over" culture differed to any great extent from those in the remaining 72 "take-over" cultures isolated from the same lake, it is considered likely that the convulsent toxin was cyanobacterial in origin.

Recent work by Sykora and Keleti (1981) has provided evidence supporting the presence of cyanobacterial endotoxins in drinking water. These endotoxins express marked similarities to those found in Gram-negative bacteria. Therefore, any of the effects witnessed on administering a mixed bacterial/cyanobacterial culture to mice could also be attributed to cyanobacterial endotoxic

rather than exotoxic activity.

Attempts to isolate a functionally axenic clone of *P. catenata* A81-15(B1) using published methods failed repeatedly. Kraus's Gamma radiation method proved to be more lytic to the cyanobacteria than to the bacteria at all dosages tested. Carmichael and Gorham's phenol method proved to be unsatisfactory with *P. catenata* and other strains of planktonic cyanobacteria. It is now thought possible that some of the problems encountered with this method in the past may have been caused by damage during suction-washing to remove the phenol.

The antibiotic enrichment method of Vaara and Vaara (1979) and Rippka (1979) proved to be unsuccessful with and without the presence of dextran 81,600. Both ampicillin and D-cycloserine affect the deposition of peptidoglycan in the walls of bacteria and cyanobacteria. The marked similarities between Gram-negative bacterial and cyanobacterial cell walls can be related to the autolysis suffered by *P. catenata* when these antibiotics were added. Parker's sulphide selection method, although unsuccessful at producing an axenic filament, did inhibit bacterial growth at the higher concentration and pH values. However, the method was a failure as far as purification was concerned. It induced stasis in the bacteria rather than their elimination. Viable colonies of *P. catenata* that appeared bacteria-free all showed contaminants after they were cored and cultured in liquid medium.

Since antibacterial agents used alone or used along with physical manipulation caused *P. catenata* to lyse, a new, gentle washing process was devised to reduce the numbers of bacteria and minimize the likelihood of putting the remainder into stasis. After a few washes however, the filaments disappeared completely, lysed, or were in stasis within a few hours of plating. The washing process, although successful in reducing the numbers of contaminating bacteria, weakened the walls of the cyanobacteria to such an extent that autolysis occurred.

A dextran (MW 81,600) at a concentration of 1% proved to be the most effective membrane protectant for *P. catenata*. The screening experiments provided evidence of very clear "cut-off" points with respect to the range of concentrations of dextran that proved beneficial to viable filament recovery. The optimum range was 0.75 to 1.5%. Above or below these limits the percentage recovery of viable filaments dropped markedly. Within such narrow limits, the protection afforded by this molecular weight and concentration range could not be simply attributed to a viscosity effect (i.e. providing sufficient support to reduce flexing or other mechanical damage). If this were the case an increase in concentration would provide a corresponding increase in the viscosity thereby increasing viability. Further, the increase in viscosity caused by above-optimum concentrations served to retain a greater number of bacteria that were not washed out.

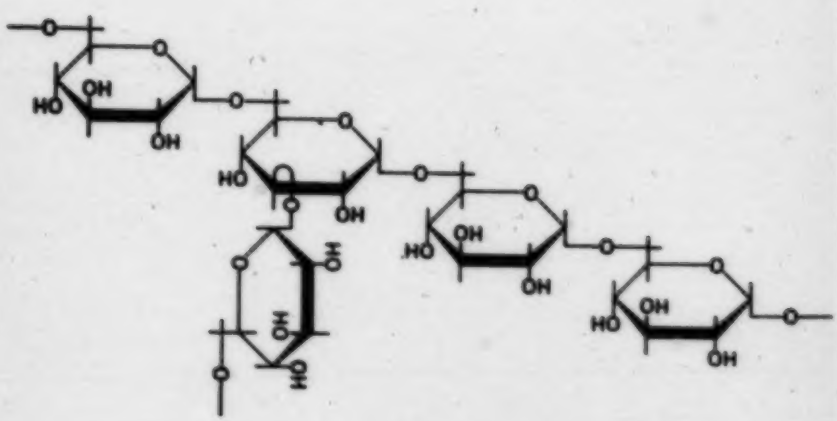
Osmotic differences were examined as the possible cause of the discrepancies recorded in the recovery rates between the various colloids and concentrations thereof. Regulating the osmolarity of each solution to the same value as that of the dextran 81,600, at 1%, did not increase the number of viable filaments remaining after the washing process, indicating that cyanobacterial lysis could not be attributed to osmotic stress.

A more plausible explanation for the optimum concentration range for dextran of appropriate molecular weight would seem to be its affinity for cell walls. At above-optimal concentrations, filaments become so heavily coated that they sediment upon the surface of the filter membrane, thereby effectively eliminating them from the recovery procedure.

The series of scanning electron micrographs showed an increasing deposition of dextran upon the filaments of *P. catenata* over the course of three hours. Although the experimental conditions used to obtain the micrographs are not strictly representative of actual washing conditions, (since the filaments do not sit in dextran for three hours), they do illustrate the fact that a coating of dextran is laid down upon the cell envelope of *P. catenata* during the washing process. Whether the encapsulating material is covalently bonded, attached by weak hydrogen bonds, or simply rests upon the surface of the cyanobacteria is unknown. However, the structure of a dextran molecule (Fig.

3) includes a number of hydroxyl groups that are free and available to bond. There is a possibility that these charged areas react with an oppositely charged portion of the cyanobacterial cell envelope to produce a bonded dextran layer.

Figure 3. Partial structure of dextran from Leuconostoc mesenteroides strain B512. It has about 95% α(1-6) glucopyranose linkages with 5% α(1-3) glucopyranose linkages at the branch points.


HO
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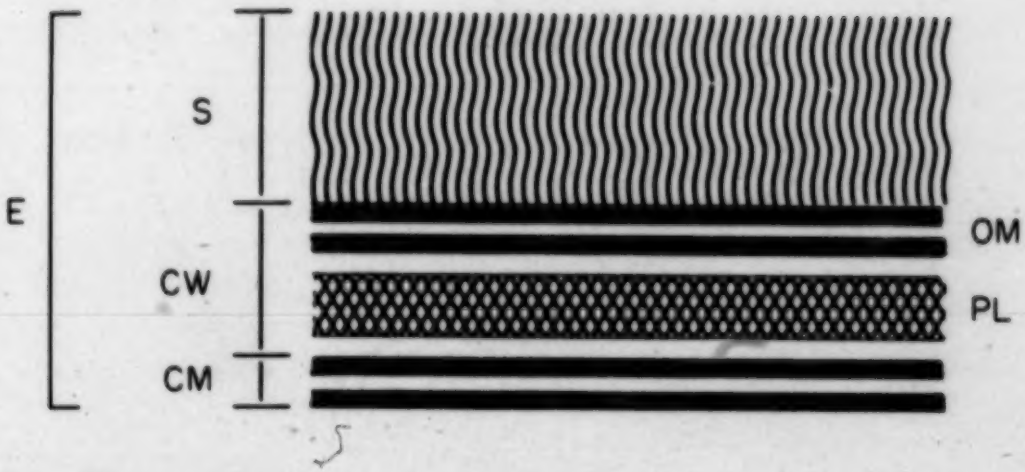
The fine structure of the cell wall of cyanobacteria is of Gram-negative type (Frank *et al*.1962, Lamont 1969, Butler and Allsopp, 1972) (Fig 4). The innermost layer of the cell wall, the peptidoglycan layer, overlays the cytoplasmic membrane. The outer membrane of the cell wall appears as a double track structure 7-10nm in diameter. In ultrathin sections it appears to be separated from the peptidoglycan layer by an electron transparent space (Butler and Allsopp, 1972). However, this space is now thought to be an artifact of the preparation as the outer membrane and the peptidoglycan layer are tightly connected in freeze-fracture preparations. A layer of similar location and appearance is found in Gram-negative bacteria and consists of lipopolysaccharide (Osborne 1963). It has been found that 2-3% of the dry weight of *Anacystis nidulans* is wall-associated lipopolysaccharide of which about 60% is carbohydrate, principally mannose, but with glucosamine, 2-amino-2-deoxyheptose, and other sugars also present (Weise *et al*.1970). This arrangement of cell wall layers (Fig 4) promotes the possibilities of both hydrogen and covalent bonds associating with the deposition of dextran.

Figure 4. Schematic representation of the cyanobacterial cell envelope.

E, cell envelope; CM, cytroplasmic membrane; CW, cell wall (PL, peptidoglycan layer + OM, outer membrane); S, external layer (slime or sheath).


E
S
CW
CM
OM
PL

Rampling (1975), using fluoresceinylthiocarbamoyl dextran (FITC-dextran) with a molecular weight of 40,100, found that it formed a loose adherent coat on erythrocyte membranes causing a decrease in the membrane flexibility. More recently, Ogiso *et al.*1977 found dextrans to produce a protecting effect against drug-induced haemolysis *in vitro*. The dextrans were found to strongly inhibit the diffusion of haemoglobin and K^+. They concluded that the protecting effect of dextran was due to the inhibition of diffusion of haemoglobin and K^+ thereby stabilizing the cellular membrane and reducing permeability.

The coating on the outer wall layers of the cyanobacteria may therefore act both as a physical shield, protecting against damage incurred during the washing process, and/or as a chemical protectant preventing increased cellular permeability by decreasing K^+ efflux.

Dextran will allow filaments to pass safely through a 10x10ml washing, a theoretical dilution of 4.1×10^{15}, which should eliminate all but the occasional bacterial cell. In reality, although the bacterial numbers are greatly reduced, they are not completely eliminated from the final washed suspension. Cyanobacteria that in various test media appear axenic may, in fact, be contaminated. Gorham *et al.*(1982) have noted that sooner or later bacteria have reappeared in what appeared to be axenic strains once they were retained in the culture collection and subcultured infrequently. A plausible explanation for this reappearance seems to be that

the cultures contain a small number of bacteria which are in stasis. Over a prolonged period of time, as the culture ages and becomes stale, these bacteria resume growth and reappear, as noted by Stanier *et al.*(1971).

After washing, the problem is primarily one of preventing or overcoming bacterial stasis at the time of coring or transfer of cyanobacterial colonies, thereby providing a truer picture of the numbers and locations of the contaminants. To this end, a new medium, P-3 washed agar, was devised to improve the growth conditions for a wider range of bacteria (Table 1). By varying the range of pH and temperatures at which test plates were incubated, conditions that caused some of the bacteria to remain in stasis were overcome.

In those plates where the viable bacterial count was virtually reduced to zero, *P. catenata*, which previously grew only under narrowly defined alkaline conditions (pH 8.5), grew well within the pH range of 7 to 9. Obviously, factors that restricted the pH-tolerance of *P. catenata* were present in the bacterized condition which were eliminated by the removal of the contaminants.

Use of the improved purification method developed for *P. catenata* with other planktonic cyanobacteria, such as *Aph. flos-aquae* and *An. sub-cylindrica*, proved to be effective in producing functionally axenic strains of these species as well. However, it should be noted that a single purification method is not likely to work for all species of

cyanobacteria, as environmental and organismic interactions are too diverse. Rather, each new species is likely to require a particular combination of specific washing conditions, culture-media, spatial separation techniques, bacteriological test media and incubation conditions to enable a functionally axenic state to be achieved.

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More about Appendix I

Appendix I has been removed from the text because of the unavailability of copyright permission. The appendix was a report of a paper in the South African Journal of Science (78:357-362) entitled "Problems Encountered in Searching for New Strains of Toxic Planktonic Cyanobacteria" by P.R. Gorham, Sean McNicholas, E.A. Dale Allen, Dept. of Botany, University of Alberta, Edmonton, Alberta.

This paper reported upon an unsuccessful search for type c toxic strains of *Aphanizomenon flos-aquae* from Lac La Nonne (80 km N.W. of Edmonton, Alberta) during the summers of 1980 and 1981. Various problems encountered in this search are described, including a high percentage of failure to obtain single colony isolates of *Aphanizomenon* caused by their gradual but complete take-over by a purple-brown species of *Pseudanabaena*.